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Pathways Involved in Formation of Mammary Organoid Architecture Have Keys to Understanding Drug Resistance and to Discovery of Druggable Targets

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Signals from the extracellular matrix (ECM) are received at the cell surface receptor, transmitted to the cytoskeletons, and transferred to the nucleus and chromatin for tissue- and context-specific gene expression. Cells, in return, modulate the cell shape and ECM, allowing for the maintenance of tissue homeostasis as well as for coevolution and adaptation to the environmental signals. We postulated the existence of dynamic and reciprocal interactions between the ECM and the nucleus more than three decades ago, but now these pathways have been proven experimentally thanks to the advances in imaging and cell/molecular biology techniques. In this review, we will introduce some of our recent work that has validated the critical roles of the three-dimensional (3D) tissue architecture in determining mammary biology, therapeutic response, and druggable targets. We describe a novel screen based on reversion of the malignant phenotype in 3D assays. We will also summarize our recent discoveries of the integration of feedback signaling for mammary acinar formation and phenotypic reversion of tumor cells in the LrECM. Lastly, we will introduce our exciting discovery of the physical linkages between the cell surface and cytofibers within a tunnel deep inside of the nucleus, enabling interaction with nuclear lamin and SUN proteins.

The microenvironment exerts control over the genome in both normal and malignant cells (Bissell and Labarge 2005; Bissell and Hines 2011). Our laboratory has spent more than three decades on understanding how the tissue microenvironment in the breast establishes and maintains tissue specificity and how this regulation is compromised in breast cancer. Such information is essential for therapeutic targeting of the tumor and the microenvironment for treatment and prevention of all forms of cancers, although the data described here deal exclusively with breast and breast cancer.

More than three decades ago, we speculated that the extracellular matrix (ECM) and nuclear machinery reciprocally and dynamically interact to regulate growth, gene expression, and phenotype of the mammary gland and other organs (Bissell et al. 1982). We postulated that the information emanating from the ECM is transferred to the cell surface receptors, is transmitted to the cytoskeleton, and is received by the nuclear matrix through the contiguous physical linkages and biochemical signaling to chromatin to allow tissue-specific gene expression. This dynamic and reciprocal signaling confers changes in cell shape, polarity, and functions that are tissue-specific and context-dependent and, ultimately, triggers the de novo synthesis of molecules. Cell and tissue polarity allow secretion of tissue-specific molecules including milk proteins and basement membrane (BM) molecules such as laminins, which, when incorporated into a structurally correct BM, will in turn give the signal for final architecture of the tissue. Almost 40 years later, the enormous advances of superresolution imaging and biomechanical/biochemical techniques have unveiled the bona fide existence of physical connections that we believe "hard-wire" the ECM to the nucleus (Wang et al. 2009; Jorgens et al. 2017). Such networks of cytoskeletal structures decisively control not only the architecture and mechanical environment but also all biological activities of the tissues. In particular, dysregulation of cytoskeletal dynamics largely contributes to malignant behavior of cancer cells (Mokady and Meiri 2015).

We summarized some of our work with human cells, three-dimensional (3D) models, reversion of the malignant phenotype, and integration of signaling, which occurs only in 3D, in a previous volume of *Cold Spring Harbor Symposia on Quantitative Biology* (Bissell et al. 2005). In this current brief review, we will not dwell on much of our earlier experimental work, which has been reviewed a number of times (Nelson and Bissell 2006; Radisky and Bissell 2006; Xu et al. 2009a; Spencer et al. 2010; Bissell and Hines 2011). Here we will introduce some of our recent work, in which we have taken advantage of old and new assays and a screen based on 3D tissue architecture to highlight the translational aspects of our studies. We will also introduce our recent studies that have shed light on how acinus is formed and how the signaling pathways integrate to close the laminin/nucleus loop in formation and maintenance of the mammary acini.

FORMATION OF MAMMARY ORGANOIDS IN 3D SUBSTRATA

A brief historical chronology of the relation between substrata, cell shape, and function was published very recently (Simian and Bissell 2017). But it has been clear from decades of literature that when cells were removed from the tissues and placed in culture, both form and function were lost (the much older but substantial literature was summarized in Bissell et al. 1982). A breakthrough occurred when Kleinman et al. (1987) analyzed the composition of what was then referred to as the EHS (Engelbreth–Holm–Swarm) matrix, a laminin-rich gel produced by a mouse tumor with striking similarity to the composition of the BM in glandular tissues. Later the gel was popularized and sold as Matrigel. The linkage between the ECM, cell shape, and gene expression was based on our finding that normal mammary epithelial cells (MECs), when isolated from the tissues and cultured in a laminin-rich gel (LrECM), form 3D alveoli-like structures. These structures are apicobasally polarized and capable of secreting milk into the lumen (Barcellos-Hoff et al. 1989). Furthermore, these structures were indeed the first reported "organoids," and our experiments and models laid the foundation for what now is generating much excitement (Simian and Bissell 2017). Of particular interest to the field are the findings that have much relevance to the fundamental properties of the tissues in vivo (i.e., apicobasal polarity of cell and tissue). This is best shown with the following work (Xu et al. 2009b): We wondered why mouse mammary cells in 2D substrata do not respond to prolactin (PR) by making milk proteins despite the fact that the cells were expressing the PR receptor. We showed that, whereas normal MECs on 2D monolayers maintain a degree of apicobasal polarity and express the PR receptors, they do not produce milk upon addition of prolactin. This is because, in these 2D-cultured cells, the PR receptors are on the basal surface that is attached to the rigid plastic substrata and not available to the medium added to the top of the monolayer (Fig. 1). Thus, in effect, the receptor and ligand are spatially segregated in 2D. On the other hand, in 3D LrECM cultures, the PR receptor is exposed to the ligand, because now the basal surface is bound to the softer ECM and becomes accessible. If the experiment is repeated with cells in PolyHEMA, a synthetic hydrogel, however, the basal surface is exposed, but the cells do not make milk when they receive PR. Such a defect indicates that, in addition to exposure of the basal receptor, we need the presence of biological molecules within the laminin-containing gel. We showed that laminin-induced polarity triggers activation of the transcription factor STAT5, which then transactivates the expression of the milk protein β-casein (Fig. 1; Xu et al. 2009b).

Because the nonmalignant cells remember to be "normal" in 3D, we explored the possibility that the tumor cells may also remember to look like a tumor in 3D! In collaboration with Ole Petersen, we developed the first human cell assay, where the two populations could be distinguished easily and robustly in 3D within less than a
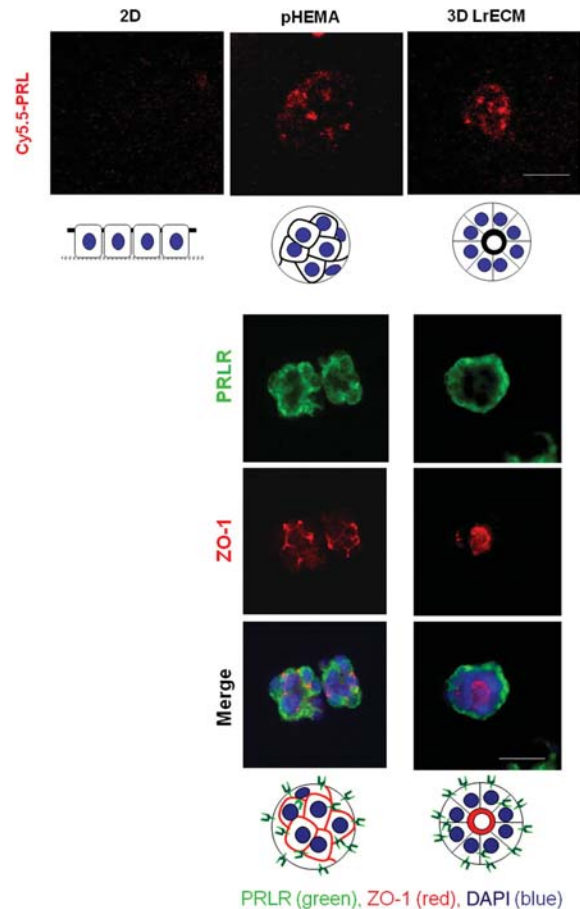

Figure 1. Prolactin receptor is exposed on the basolateral surface when mammary epithelial cells form apicobasally polarized colonies in three-dimensional (3D) laminin-rich extracellular matrix (LrECM). (*Top*) Representative micrograms of mouse mammary epithelial cells EpH4 incubated with Cy5.5-labeled prolactin (PRL). Cells were cultured in 2D plastic, on poly-HEMA (pHEMA), or in 3D LrECM. (*Bottom*) EpH4 cultures on pHEMA or LrECM were stained for prolactin receptor (PRLR), apical marker ZO-1, and nuclei (DAPI). Scale bar, 25 μm. (Reprinted, with permission, from Xu et al. 2009b, the Rockefeller University Press.)

week instead of months in mice (Petersen et al. 1992). Thus, 3D phenotype could be used to distinguish normal from malignant cells. We took advantage of the isogenic HMT3522 breast cell lines that were established from reduction mammoplasty and propagated from nonmalignant cells (S1), to premalignant cells (S2), and after many tries, to malignant cells (T4-2) (Briand et al. 1987). We showed that, whereas nonmalignant S1 cells form quiescent, apicobasally polarized spheroids in 3D LrECM cultures, their malignant counterparts, T4-2 cells, form proliferative, disorganized masses in the same culturing condition (Fig. 2; Petersen et al. 1992; Weaver et al. 1997; Lee et al. 2007). Such differential response to LrECM between normal and malignant cells to the same cues suggested that the pathways responding to laminin 111 (LN)-1 are altered in malignant cells (Petersen et al. 1992).
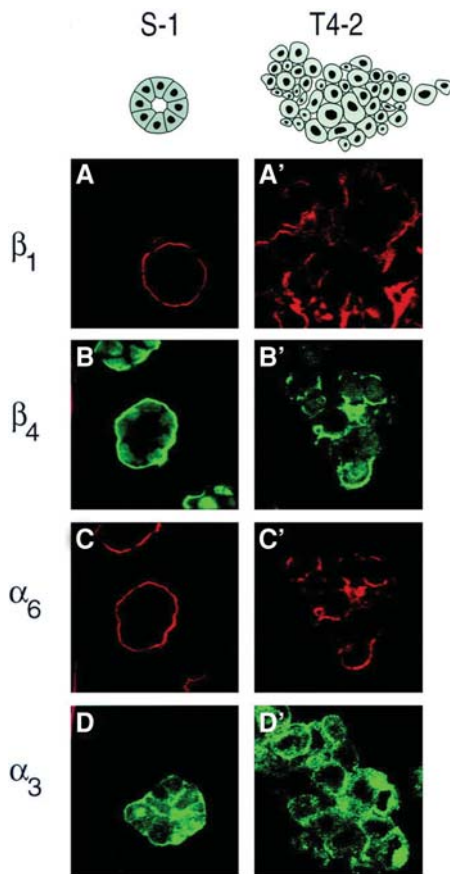
Figure 2. Normal and malignant mammary epithelial cells form distinct colonies in three-dimensional (3D) laminin-rich extracellular matrix (LrECM) cultures. Immunofluorescence characterization of the HMT-3522 cells in 3D cultures after 10−12 d inside the Engelbreth−Holm−Swarm (EHS) matrix. ($A−D$) S-1 acini and ($A'−D'$) T4-2 colonies immunostained β1- (A and A'), β4- (B and B'), α6- (C and C'), and α3-integrin (D and D'). Note that β1-, β4-, and α6-integrins were targeted to the cell−ECM junction in the S-1 acini ($A−C$), whereas in T4-2 colonies ($A'−C'$) this polarized-basal distribution was lost. S-1 acini showed basolateral α-3 integrins (D), whereas T4-2 colonies (D') showed disorganized plasma membrane and cytosolic expression of this integrin. (Reprinted, with permission, from Weaver et al. 1997, the Rockefeller University Press.)

We explored the potential mechanisms by which nonmalignant MECs and breast cancer cells (both from the same individual) show such profound phenotypic differences in LrECM. To address the mechanism of growth factor−independent, autonomous growth of cancer cells, we measured the levels of receptors expressed on the surface of normal versus malignant cells and found that, although these cells appeared to have similar receptors, the level of integrins, including β1 and α/β6, as well as epidermal growth factor receptor (EGFR) were elevated between four- and sixfold in malignant cells in 3D LrECM (Fig. 2; Weaver et al. 1997). Remarkably, reduction of a number of these pathways to the level of nonmalignant S1 led to a phenotypic reversion of morphology and growth, allowing T4-2 cells to resemble the S1 cells but only in 3D (summarized in Bissell et al. 2005;

see below for more details). To get insight into events that led to conversion of S1 cells to premalignant S2 and to malignant T4-2, we found that once EGF, the only growth factor in the medium, was removed, cells became adapted to the absence of EGF by up-regulating the expression of a matrix metalloproteinase (MMP), TACE. TACE promotes shedding of an autocrine factor, amphiregulin (ARG), from the cell surface. ARG then binds the EGFR on cancer cells to induce oncogenic signaling, without requirement of exogenous EGF (Kenny and Bissell 2007).

We asked: How is it that the nonmalignant cells, with many mutations, deletions, and amplifications, know to still form an acinus in 3D, but once malignant, they no longer do? In a data-rich and ambitious paper (Rizki et al. 2008), we summarized years of work and showed that the structure of the acini is compromised even before malignancy sets in (please see the upper panel in Fig. 5). To understand how each step is executed to induce formation of a complete acinus in the LrECM (pure laminin 111, as a 20% rat tale collagen gel can substitute for Matrigel and, of course, it is much better defined) (Alcaraz et al. 2008), we started with putting single primary human breast cells from reduction mammoplasty, or even a breast cell line inside the gel, and imaged live cells continuously for 10 days when all cells formed acini. Remarkably, the cells began to rotate continuously through a process we termed "coherent angular motion" (CAMo) (Tanner et al. 2012). Comparing cellular motions between normal MECs and breast cancer cells, we found that nonmalignant cells undergo CAMo to form a polarized spherical cluster, whereas they are tightly tethered to each other and remain polar during cell division. The coherent rotating movement of acinus forming MECs resembles that of *Drosophila* follicles during embryogenesis (Haigo and Bilder 2011). In contrast, malignant cells undergo a random movement and form a disorganized mass as they divide and their interactions are quite loose (Fig. 3; Tanner et al. 2012). The ability of nonmalignant MECs to undergo CAMo was dependent on the presence of intact cell−cell junctions, because inhibition of PAR3 (tight junction and polarity) or E-cadherin (adherin junctions) completely abrogated CAMo in nonmalignant MECs. This result again highlights the fact that the same mechanical and biochemical cues from the ECM are transduced differently in normal versus malignant cells, leading to different cellular motions and, thus, formation of different tissue structures. When tumor cells are treated to revert to a normal phenotype, the movement becomes coherent again (Tanner et al. 2012).

By using the 3D LrECM cultures, we explored whether altering the extracellular growth signaling pathways could convert the 3D phenotype of malignant cells to that of normal cells. We discovered that suppression of extracellular growth signals, including ECM or growth factor receptors (e.g., β1 integrin or EGFR), induces tumor reversion and restores a normal-like, polarized, and quiescent phenotype in malignant cells (Figs. 2, 4, and 5; Weaver et al. 1997, 2002; Bissell et al. 2005; Lee et al. 2007, 2012; Beliveau et al. 2010). We have found
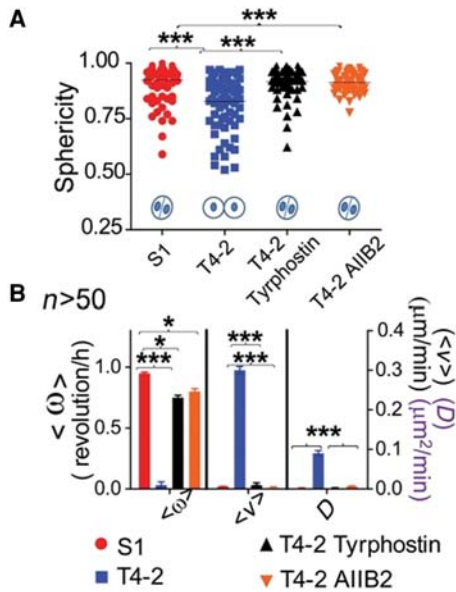
Figure 3. Different movements of normal versus malignant mammary epithelial cells during morphogenesis in three-dimensional (3D) laminin-rich extracellular matrix (LrECM). (*A*) Comparison of cell adhesion measured by sphericity of the cell aggregates. (*B*) Graph depicting different types of motility: ω, angular motion; *v*, linear displacement; *D*, diffusion; $^*P <$ 0.05; $^{**}P < 0.01$; $^{***}P < 0.0001$. (Reprinted, with permission, from Tanner et al. 2012, the National Academy of Sciences of the United States of America.)

more than a dozen different pathways involved in tumor reversion (Weaver et al. 1997, 2002; Bissell et al. 2005; Lee et al. 2007, 2012), suggesting the presence of common regulators that integrate these different signals to produce essentially the same architecture—namely, a reverted structure that resembles a nonmalignant acinus. For example, up-regulation of integrins, EGFR, and other growth pathways converge on activation of MMP-9 (Beliveau et al. 2010). Inhibition of any of these pathways to the level of S1 (nonmalignant) inactivates MMP-9, leading to phenotypic reversion (Beliveau et al. 2010).

The phenomenon of tumor reversion provides us with a robust tool to determine critical signals that are involved in formation of normal tissue architecture and are dys-

regulated in cancer. Such an approach allows us to identify novel tumor suppressors or oncogenes, based on cells' ability or inability, respectively, to undergo phenotypic reversion (Chen et al. 2009; Lee et al. 2012). Utilization of tumor reversion also contributes to development of a new therapeutic regimen that corrects microenvironmental cues (Weaver et al. 1997; Wang et al. 1998; Kenny and Bissell 2003; Beliveau et al. 2010; Lee et al. 2012; Onodera et al. 2013).

USE OF 3D MORPHOGENESIS ASSAY TO IDENTIFY NOVEL TUMOR SUPPRESSORS AND "ONCOGENES" AND TO DISCOVER POTENTIAL TREATMENTS FOR BREAST CANCER

It has long been known that early pregnancy may have protective effects for breast cancer (Britt et al. 2007). The underlying mechanism, however, is still speculative. We postulated that such protection is mediated by formation of mammary alveoli during pregnancy that may produce certain antitumor factors to suppress the growth of premalignant and malignant cells in the mammary gland. Whereas this may appear to be a wild and crazy guess, evolution has had to protect the continuation of the species by developing lush and intricate mechanisms that we already know are built-in to assure that babies are conceived and thrive once born. So a healthy and robust acinar structure is necessary for producing milk and secreting it into the lumen so the baby can be properly fed! We had already developed a robust, physiologically relevant model to examine how acini are formed and how they produce milk vectorially (Aggeler et al. 1991). To test this possibility, we used 3D LrECM cultures that allow for formation of polarized acini recapitulating mammary alveologenesis in vivo during pregnancy. We collected the conditioned media (CM) of nonmalignant human MECs during acinar formation and used the media to culture malignant MECs in 3D LrECM. We found that tumor cells underwent phenotypic reversion when cultured in the presence of the CM of acinar-forming nonmalignant MECs. We then analyzed the components of the CM by mass spectrometry and identified IL25 as the major factor that showed antitumor effects (Furuta et al.
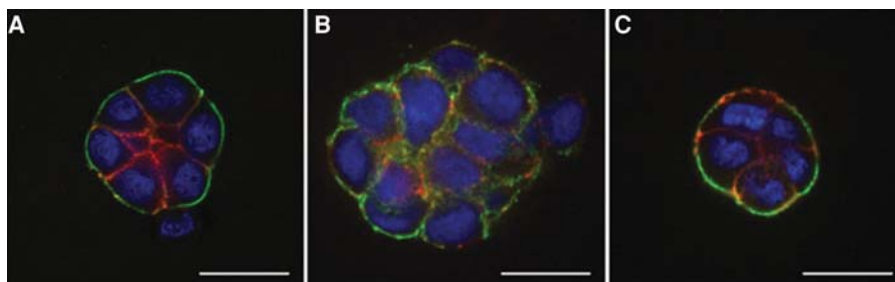

Figure 4. Epidermal growth factor receptor (EGFR) inhibition restores S1-like quiescent, polarized three-dimensional (3D) structures of malignant T4-2 cells. (*A–C*) HMT-3522 S1 (*A*), HMT-3522 T4-2 (*B*), and HMT-3522 T4-2 treated with an EGFR inhibitor, AG1478 (*C*) were cultured in the 3D on-top assay for 4 d. Colonies were then extracted and immunostained against α_6 integrin (green) and β-catenin (red). Nuclei were counterstained with DAPI (blue). Confocal sections through the centers of the colonies are shown. Scale bar, 20 μm. (Reprinted, with permission, from Lee et al. 2007, Nature Publishing Group.)
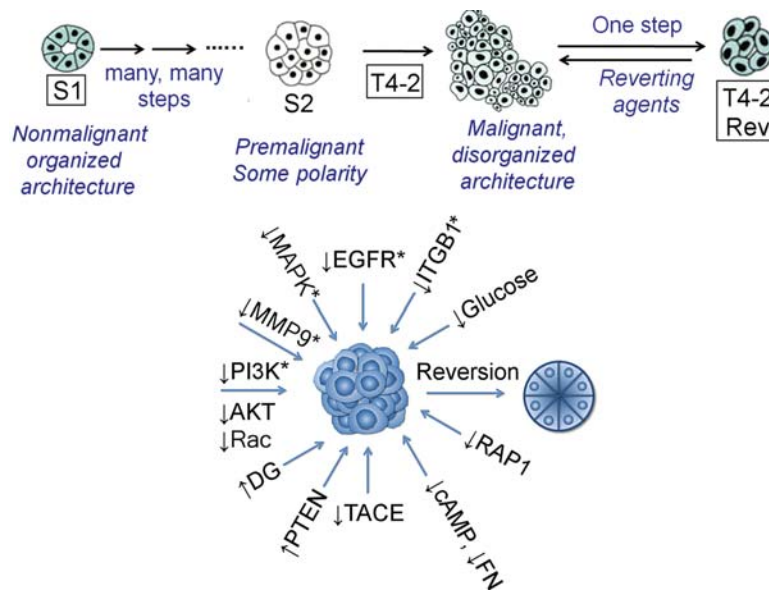
Figure 5. Inhibition of diverse signaling pathways leads to phenotypic reversion of tumor cells. (*Top*) Scheme of tumor progression and reversion. (*Bottom*) Different means to induce tumor reversion. The methods used in gene and microRNA expression profiling are indicated by an asterisk (*). EGFR, epidermal growth factor receptor; ITGB1, integrin beta 1; cAMP, cyclic adenosine monophosphate; FN, fibronectin; PTEN, phosphatase and tensin homolog; DG, dystroglycan; PI3K, phosphoinositide 3-kinase; MMP9, matrix metalloproteinase 9; MAPK, mitogen-activated protein kinase.

2011). We further explored the mechanism of antitumor effect of IL-25 and found that the majority of cancer cells analyzed up-regulated the receptor IL25R, which could bind both IL25 and IL17B. IL-25-IL25R interactions activated death receptor signaling leading to caspase activation. In contrast, IL17B–IL25R interaction led to activation of NF-κB and proliferative pathway. Interestingly, nonmalignant MECs produced IL25 only during acinar formation, whereas cancer cells produced only IL17B. Furthermore, IL25R is present only in cancer cells. These results uncover a novel mechanism of normal breast tissue exerting natural defense against cancer cells. This unexpected discovery was possible because of normal cells' ability to undergo in vivo–like tissue morphogenesis in 3D LrECM cultures (Fig. 6; Furuta et al. 2011).

We have often wondered why so many cancer patients, despite high levels of EGFR in their primary tumors, do not respond to a number of current tyrosine kinase inhibitors (TKIs) such as lapatinib, and why so often they respond and within a short period they become resistant? We hypothesized that there may be additional intermediates in the EGFR and related pathways that still have not been discovered because many of the screens rely only on monolayer cultures and normal cells are hardly ever included in toxicity studies. The 3D LrECM cultures can be "used" to test these ideas. We often had noticed that a small fraction of T4-2 cancer cells in our 3D assays displayed resistance to signaling inhibitors that induce phenotypic reversion in the bulk of tumor cells. We suspected that the existence of these "resistant colonies" to our EGFR inhibitors, "reverting agents," was due to up-regulation of some novel oncogenic intermediate in the resistant cells. We generated the cDNA library from

malignant T4-2 cells, overexpressed the library in T4-2 cells, and determined which cDNA made T4-2 cells resistant to the EGFR inhibitor in 3D cultures. We identified FAM83A as the suspected oncogene that confers resistance to EGFR inhibitor (Fig. 7; Lee et al. 2012). During a chance encounter, we discovered that another laboratory, using an entirely different screen by using breast cell lines from Martha Stampher's 184 breast cancer progression series, had identified FAM83B in their screen (Cipriano et al. 2012). In the ensued collaboration, together we have uncovered that FAM83A and FAM83B are among the eight members of a new class of onco-
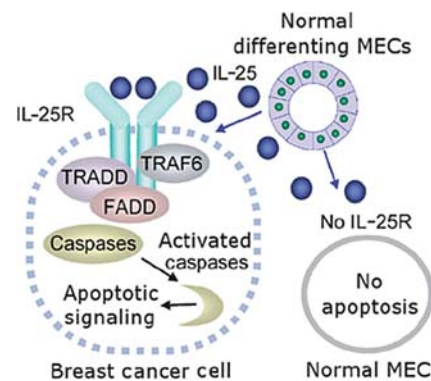

Figure 6. Schematic for the cytotoxic activity of IL-25 specific to breast cancer cells that express IL25R. Nonmalignant mammary epithelial cells (MECs) do not express IL-25R and are resistant to apoptosis induced by IL-25. Breast cancer cells that express IL-25R are susceptible to IL-25-induced apoptosis. (Reprinted, with permission, from Furuta et al. 2011, AAAS.)
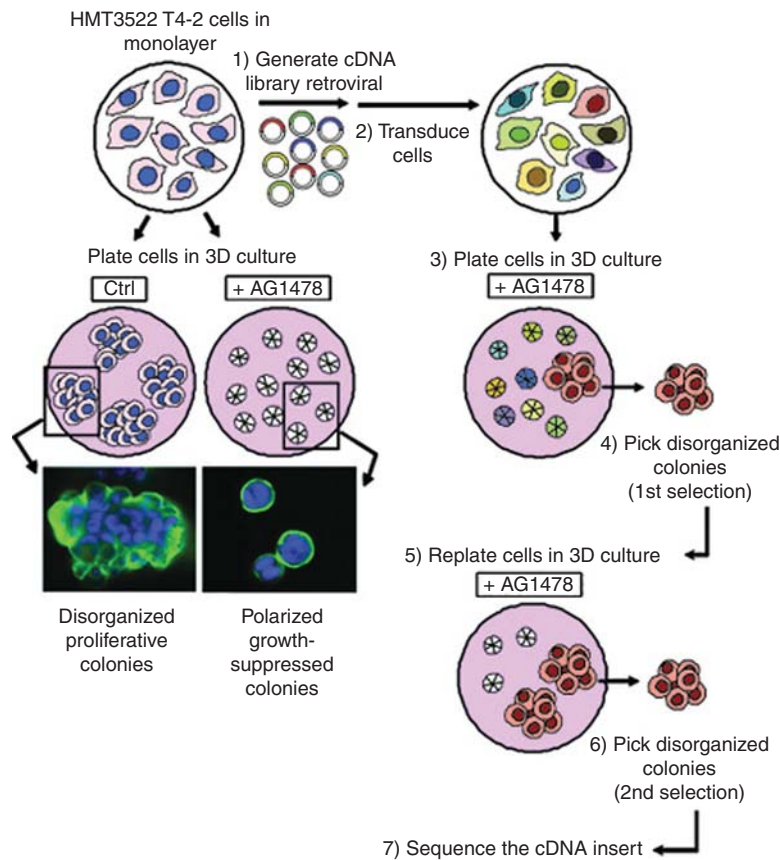
Figure 7. Process for the screen to identify FAM83A. Treatment of T4-2 cells with a small-molecule epidermal growth factor receptor (EGFR) inhibitor, AG1478, induced reversion to a polarized acinar phenotype in three-dimensional (3D) laminin-rich extracellular matrix (LrECM) culture, as detected by α_6 integrin staining (green) to delineate basal polarity (*left*); after transduction with the cDNA library, a subpopulation of cells disorganized in 3D culture after AG1478 treatment was isolated, expanded, and searched for the cDNA insert (*right*). (Reprinted, with permission, from Lee et al. 2012, American Society for Clinical Investigation.)

genes, FAM83A through FAM83H. We found that FAM83A interacts with both c-RAF and the p85 subunit of phosphoinositide 3-kinase (PI3K), facilitating their activation. We further showed that treatment of mammary tumors with lapatinib, a clinically used EGFR and EGFR2 inhibitor, while killing the majority of cells, up-regulated FAM83A expression in a small fraction of surviving cells in vivo leading to resistance to TKIs. This result strongly supports that FAM83A up-regulation is a cause of lapitinib resistance of breast tumors (Lee et al. 2012). These FAMs indeed are highly expressed in many tumor types including lung, stomach, and breast and are druggable targets in the pathways of EGFR and PI3K. They are induced to high levels in vivo after mice with human tumors are given lapatinib (Snijders et al. 2017). Such findings also suggest that the degree of phenotypic reversion of tumor cells in 3D LrECM cultures could be used as the index of the efficacy of cancer treatment in vivo.

We have examined also the contribution of 3D LrECM environment to chemo-resistance of breast cancer cells. We treated different human epidermal growth factor receptor 2 (HER2)-amplified breast cancer cell lines (AU565, SKBR3, HCC1569, and BT549) cultured in either 2D plastic or 3D LrECM with a clinically used HER2-targeting drug (trastuzumab, pertuzumab, and lapatinib). We found that the drug responses of these cell lines differ significantly between 2D and 3D conditions because of differential activation of PI3K and mitogen-activated protein kinase (MAPK) pathways in response to the drug (Weigelt et al. 2010). The cotreatment of $\beta1$ integrin inhibitory antibody (AIIBII), however, augmented drug effect of all the conditions, suggesting that integrin-mediated cell–ECM anchorage had reduced drug efficacy for these cells tested (Weigelt et al. 2010).

To apply this finding to therapy, we xenografted different breast cancer cell lines subcutaneously into immunocompromised mice and applied $\beta1$ integrin inhibitory antibody (AIIBII). We observed significant suppression of tumor take and growth by AIIBII. In contrast, normal breast tissue, as well as nonmalignant cells undergoing acinar morphogenesis in 3D LrECM cultures, were not affected by AIIBII (Park et al. 2006). This result suggests that polarized nonmalignant cells were resistant to the growth inhibitory effects of the $\beta1$ integrin blocking antibody. Further, the study strongly indicates that the $\beta1$ integrin inhibitory antibody could be used as a cancer-specific therapy with minimal side effects on both normal

cells in 3D cultures and in vivo (Park et al. 2008). We tested the efficacy of adjuvant use of AIIBII for radiation treatment of xenografted breast cancer cells. We found that AIIBII, which drastically inhibited AKT activation, significantly enhanced radiation-induced apoptosis of breast cancer cells. This allowed the radiation dosage to be reduced by fourfold (8 Gy to 2 Gy) to obtain the same apoptotic effect (Park et al. 2008). These results suggested strongly that targeting the β1 integrin-mediated cell–ECM linkage could be a promising method for breast cancer treatment. The results of using the humanized inhibitory antibodies against β1 integrin are encouraging.

LINKAGE BETWEEN THE ECM AND NUCLEUS

Three-dimensional environments that enable tissues to express tissue-specific genes for functional differentiation have allowed us also to unravel important signaling pathways that are integrated to define the tissue architecture.

One of the first evidences of the linkage between the ECM and the nucleus from our laboratory was our finding of the ECM-response element. The promoter region of bovine β-casein gene was found to contain 160-bp bovine casein response element (BCE)-1 transcriptional enhancer at ~1.5 kb upstream of the transcription start site. BCE-1 controls transcriptional activation of the milk protein, β-casein gene (Schmidhauser et al. 1992). BCE-1 is activated not by binding of transcription factors that have consensus sequences within that region but by removing chromatin inactivation exerted by histone deacetylase in response to the ECM protein laminin (Streuli et al. 1995; Myers et al. 1998).

Another example is Seid et al.'s finding of the promoter region of LpS1 gene (called proximal G-string) in sea urchin that is activated in response to collagen. A collagen cross-linking inhibitor (β-aminopropionitrile) impairs activation of proximal G-string, whereas a mutation in that region causes constitutive activation, suggesting that G-string activation is mediated by removal of repression in response to collagen (Seid et al. 1997).

Lastly, we recently have discovered that the promoter region of the laminin α3 chain (LAMA3) of laminin 332 (also known as laminin 5) is activated by p53 in response to the ECM laminins in the normal breast. The LAMA3 gene promoter contains numerous (more than 20) consensus sequences of p53 within 1 kb upstream of the transcription start site, whereas inactivation of p53 abrogates LAMA3 transcription. LAMA3 expression is essential for formation of normal mammary acini (milk secretory unit) in 3D ECM cultures (S Furuta and MJ Bissell, in prep.).

Another evidence for the ECM–nucleus linkage is the shuttling of nuclear actin between the nucleus and cytosol in response to ECM cues. Although polymeric actin is an essential cytoskeletal component in the cytosol, monomeric actin is shuttled between the cytosol and nucleus (Sen et al. 2015). In the nucleus, actin plays critical roles in nuclear matrix organization, chromatin remodeling,

mRNA processing, and transcriptional regulation (Visa and Percipalle 2010). Nuclear actin is part of the nuclear matrix (Kiseleva et al. 2004) and interacts with the nuclear lamina, pre-mRNA splicing proteins, and chromatin remodeling complexes for transcriptional activation of tissue-specific genes (Clubb and Locke 1998; Lamond and Spector 2003; Gruenbaum et al. 2005; Farrants 2008; Visa and Percipalle 2010; Sen et al. 2015). Conversely, polymeric actin in the nucleus is thought to play roles in regulating nuclear shape and positioning in response to mechanical stress (Plessner et al. 2015). Actin translocation into the nucleus is facilitated by importin 9 in response to stress (e.g., heat shock) (Nishida et al. 1987; Pendleton et al. 2003; Dopie et al. 2012; Belin et al. 2015). Conversely, we found that, when cells are exposed to laminin 111 or LrECM, nuclear actin is transported from the nucleus to the cytosol (Fig. 8; Spencer et al. 2011). This destabilizes RNA polymerase II/III complex at transcription site and inhibits transcription and DNA synthesis, allowing for cells to enter the quiescent state for functional differentiation (Spencer et al. 2011). In contrast, overexpression of actin harboring nuclear localization signal inhibits export of nuclear actin and growth arrest (Spencer et al. 2011). Translocation of nuclear actin to the cytosol is mediated by exportin 6 (Stüven et al. 2003). Exportin 6 levels determine cells' abilities to translocate nuclear actin. Exportin 6 expression is elevated in normal breast epithelial cells in response to laminin or LrECM, triggering translocation of nuclear actin. In contrast, laminin-responsive exportin 6 up-regulation is abrogated in breast cancer cells, impairing actin translocation (A Bruni-Cardoso and MJ Bissell, in prep.).

Most recently, we have elucidated the detailed mechanism that integrates different signaling cascades for mammary morphogenesis using the 3D LrECM system. These signals emanate from reciprocal interactions between different cellular and acellular components, forming integrated feedback loops (Xu et al. 2009a; Mammoto and Ingber 2010; Patwari and Lee 2010; Basson 2012). We compared gene expression profiles of nonmalignant S1, malignant T4-2 cells, and T4-2 cells reverted with six different reverting agents, including inhibitors against EGFR, β1 integrin, and MMP9, after culturing in LrECM for 10 days. We found that there is a cluster of 70 genes that were all up-regulated in T4-2 cells, but down-modulated in S1 and "reverted" T4-2 cells (Bissell et al. 2005). To test whether these genes are under the control of common transcriptional regulators, we analyzed the consensus sequences in the promoter regions of these genes. We found that all of them possess multiple binding sites of the RelA subunit of NF-κB. NF-κB was highly up-regulated in malignant T4-2 cells and activated the expression of these 70 genes. We then inhibited NF-κB in T4-2 cells and found that this led to phenotypic reversion of malignant cells in LrECM culture (Becker-Weimann et al. 2013). This result suggests that NF-κB plays a critical role in disintegration of diverse signaling involved in mammary morphogenesis and must be silenced to allow phenotypic reversion in response to LrECM. To further
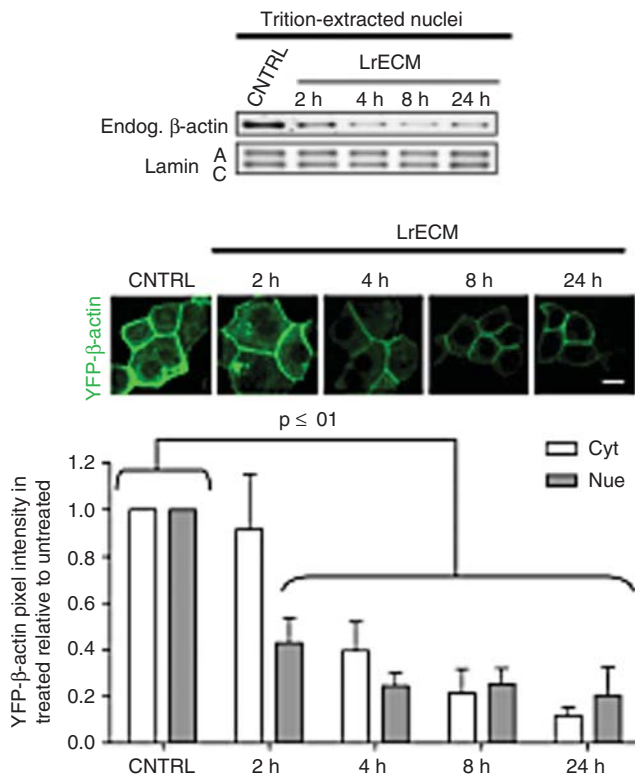
Figure 8. Laminin-rich extracellular matrix (LrECM) triggers the export of actin from the nucleus in normal mammary epithelial cells. (*Top*) Western blot analysis of actin level in the nucleus after addition of LrECM. (*Middle*) Micrograms of cells expressing yellow fluorescent protein (YFP)-β-actin. (*Bottom*) Quantification of the YFP signal in the cytosol versus nucleus after addition of LrECM. Cyt, cytosol; Nuc, nucleus. (Reprinted, with permission, from Spencer et al. 2011, The Company of Biologists Limited.)

explore the detailed molecular mechanism of the signal integration, we performed microRNA expression profiling of S1, T4-2 cells, and T4-2 cells reverted with the same conditions as those used for gene expression profiling (Fig. 5). By combining the results of gene expression profiling and microRNA expression profiling, we identified a group of microRNAs that could regulate the cluster of genes identified in gene expression profiling. While studying the mechanism of regulation of these microRNAs, we unraveled the signaling circuit that is activated in response to the basement membrane proteins laminins and responsible for the morphogenetic program of MECs (Fig. 9) (S Furuta and MJ Bissell, in prep.). We found that LrECM treatment in nonmalignant MECs activates p53 as the earliest responses (within 30 min), which then binds and transactivates the expression of LAMA3 (α3 chain of laminin-332) for exerting breast tissue specificity. Concomitantly, activated p53 up-regulates expression of a group of microRNAs that inactivate the expression of MMP9, the enzyme that degrades laminins, for protection of de novo synthesized laminins. Up-regulated laminin-332 then repeats the whole morphogenetic signaling cascade (S Furuta and MJ Bissell, in prep.). During proper morphogenesis, cytoskeletons are reorganized, and cell junctions and polarity are reinforced, leading to formation of apicobasally polarized structures. We showed that de novo synthesis of laminin-332 by MECs is the ultimate determinant of these processes. We also showed that this mechanism, conversely, is defective in cancer cells, which accounts for their inability to form quiescent, polarized structures in LrECM. Nevertheless, modulation

of signals "downstream" of p53 (e.g., NF-κB, EGFR, β1 integrin, or MMP9) in cancer could reciprocally restore the upstream p53 activation, allowing for resumption of a normal morphogenetic program (Fig. 9). The overall findings of this study not only attest to the fact that morphogenetic signaling cascades establish integrated feedback loops but also show the rapid and intimate communications between the ECM and nucleus (S Furuta and MJ Bissell, in prep.).

Lastly, we visualized cytoskeletal structures that serve as the physical and mechanical connections between the ECM and nucleus using superresolution imaging techniques. Recent studies by other groups revealed the detailed mechanism of mechanotransduction that transmits the ECM stimuli to the nucleus. It is found that force received at the cell–ECM interphase is transmitted, through cytoskeletons and then to the linker of nucleoskeleton and cytoskeleton (LINC) complex located between the outer and inner nuclear membranes (Chang et al. 2015). LINC mechanically links cytoskeleton to the nucleus (Osmanagic-Myers et al. 2015) and plays roles in force transduction, as well as DNA damage repair and chromosome anchoring (Chang et al. 2015). Defects of LINC lead to nuclear deformation and impaired polarization of cells (Osmanagic-Myers et al. 2015). To further analyze these cytoskeletal structures deep inside of the nucleus, we collaborated with Manfred Auer and visualized the ultrastructures of mammary acini formed by nonmalignant MECs in LrECM cultures. Our work showed that, within the nucleus, cytoskeletal elements comprised of actin and keratin invaginate and
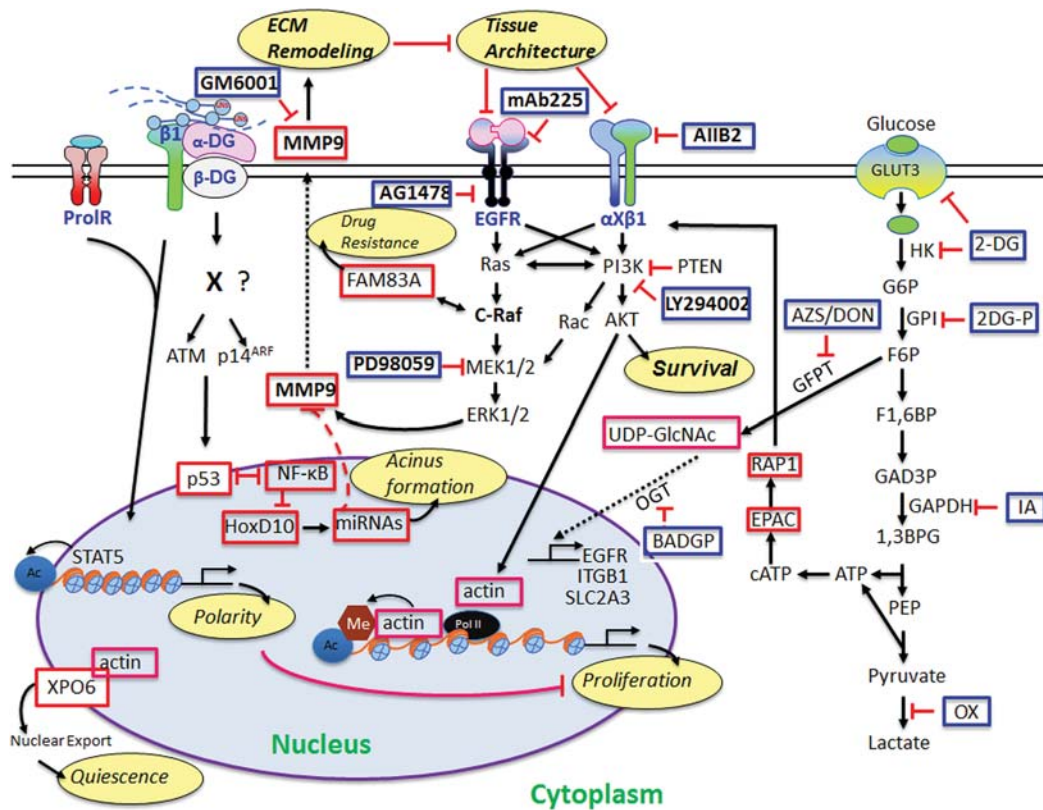
Figure 9. Scheme of signal integration and feedback loops during mammary acinar formation and tumor cell reversion. The different signaling cascades that we have reported are involved in acinar formation and tumor reversion are shown. ECM, extracellular matrix; MMP9, matrix metalloproteinase 9; DG, dystroglycan; EGFR, epidermal growth factor receptor; ITGB1, integrin beta 1; PI3K, phoshoinositide 3-kinase; PTEN, phosphatase and tensin homolog; cATP, cyclic adenosine triphosphate; PEP, phosphoenolpyruvate; GAPDH, glyceraldehyde 3-phosphatedehydrogenase; GPI, glucose 6-phosphate isomerase. (Courtesy of Sun-Young Lee.)
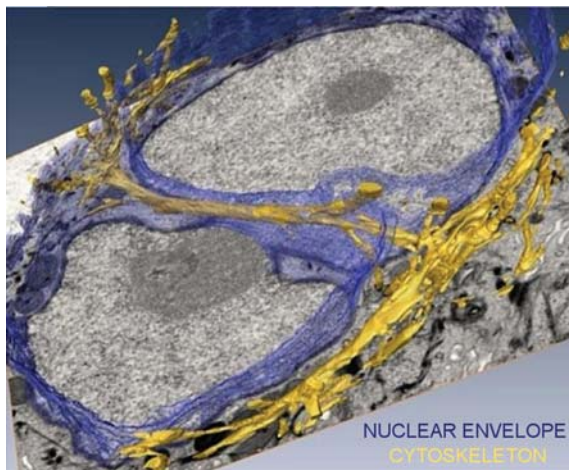

Figure 10. Cytoskeletal structures (yellow) in the nucleus are completely enclosed by the nuclear envelope (purple) and transverse the nuclear tunnel. The image was captured by serial block face scanning electron microscopy. Note that this image shows the ultrastructure of a single nucleus. (Reprinted, with permission, from Jorgens et al. 2017, The Company of Biologists Limited.)

transverse the nuclear tunnel. This structure ultimately forms the bridge between the adhesion complexes on the cell surface (desmosomes and hemidesmosomes) and nucleus (Fig. 10; Jorgens et al. 2017). These findings provide the visual support for the physical and mechanical links from the cell surface to the deep inside of the nucleus. The finding serves as the evidence for the physical path of dynamic reciprocity between the ECM and the nucleus by which ECM stimuli are transmitted into the nucleus for tissue-specific and context-dependent gene expression. Future investigation of whether this system is impaired in cancer cells is of great importance and is under investigation in our laboratory.

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